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Document Number 5

   Entry 5 of 16   File: USPT   Oct 27, 1998

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TITLE: Adipogenic differentiation of human mesenchymal stem cells

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INVENTOR-INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY |
| Pittenger; Mark F. | Severna Park | MD | N/A | N/A |

ASSIGNEE INFORMATION:

| NAME | CITY | STATE | ZIP CODE | COUNTRY | TYPE CODE |
| Osiris Therapeutics, Inc. | Baltimore | MD | N/A | N/A | 02 |

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U.S. PATENT DOCUMENTS

| PAT-NO | ISSUE-DATE | PATENTEE-NAME | US-CL |
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PRIMARY-EXAMINER: Saunders; David

ASSISTANT-EXAMINER: VanderVegt; F. Pierre

ATTY-AGENT-FIRM: Herron; Charles J. Olstein; Elliot M.

ABSTRACT:

A composition which comprises human mesenchymal stem cells which have the potential to differentiate into cells of more than one connective tissue type and a composition which induces cells from the mesenchymal stem cell population to differentiate into the adipogenic lineage, and a process for inducing such differentiation. The composition for inducing such differentiation comprises a glucocorticoid and a compound which stimulates cAMP production or inhibits cAMP degradation (such as a phosphodiesterase inhibitor). The process can further include isolating the adipocytes from remaining hMSCs.

14 Claims, 17 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 9

BRIEF SUMMARY:

This invention relates to adipocytes and more particularly to producing adipocytes from human mesenchymal stem cells.

Adipose tissue provides an energy storage reserve for the body in the form of triglycerides and this tissue can release free fatty acids when caloric intake falls below metabolic needs. In response to increased dietary intake, the body will normally automatically increase energy expenditure through activity to maintain an energy balance. Energy can also be released as heat. Adipose tissue is intimately involved in the maintenance of body temperature through brown adipose tissue and energy storage through white adipose tissue. There are normal energy regulation pathways that balance dietary intake with metabolic activity largely mediated through the hypothalamus. It is now also apparent that the adipocyte plays

an active role in this process and likely produces molecules that serve to feed back and effect regulation of triglyceride metabolism.

The two types of adipose tissue, brown and white, carry out very different roles in the body. White adipose is designed to store excess caloric intake while brown adipose tissue uses a unique system to syphon off excess calories and use it to generate body heat. The heat is generated in the mitochondria of brown adipose where oxidation of substrate is utilized to create a hydrogen ion gradient that is then collapsed in a regulated fashion generating heat instead of ATP. It has been shown that transgenic animals that lack brown adipose maintain efficient metabolism, are obese and continue to overeat (Lowell et al, 1993). Other rodent studies have also shown a link between obesity, continued overeating and a sensitivity to cold, suggesting a connection to the sympathetic nervous system (Friedman and Leibel, 1992).

Imbalance in energy metabolism in the body leads to several diseased states, most notably obesity and obesity-induced diabetes and these can be described as dysfunctions of energy storage tissues. A mutation in mice that leads to obesity was identified in 1950 (Ingalls et al., 1950) and the gene was recently identified by positional cloning. The product of the ob gene is a 16,000 mw protein named leptin or OB protein. Leptin is produced only by adipocytes and is a hormone which regulates the hypothalamus. A mutation has been identified in the of mouse that results in premature termination of mRNA translation such that no functional leptin protein is made (Zhang et al. 1994). The role of leptin in regulation of lipid metabolism is an area of intense research. Recent published investigations include studies of the upstream promoter elements found adjacent to the ob gene which have been shown to bind C/EBP (or CCAAT/enhancer binding protein) (Yeh et al, 1995 and Hwang et al., 1996). Having a model experimental system for in vitro adipogenesis of human cells would provide for discoveries in this area.

Recently it has been reported that leptin may serve as a hormone that regulates fertility and may be the link between appropriate body weight and reproductive physiology (Chehab et al. 1996). Both underweight and overweight women have difficulty in conceiving and this is likely associated with hormonal imbalance in the body of these individuals. The connection between body weight, fertility and the leptin produced by adipocytes has been suspected and now tested in mice. When obese mice, which normally do not produce offspring without transplanting the ovaries to surrogate females, were injected with leptin, there body weight fell dramatically and they were able to give birth to their own litters (Chehab et al, 1996).

A variety of cell types have been shown to produce lipid containing vesicles under specific culture conditions. For example, mouse 3T3-L1 cells derived from NIH 3T3, an immortalized mouse cell line, can be grown and cultured as a

fibroblastic cell. However, after exposure to dexamethasone and methyl-isobutylxanthine, the cells undergo differentiation which results in the production of intracellular lipid-containing vacuoles (Spiegelman and Green, 1981). Rat marrow stromal cells have been shown to undergo both osteogenic and adipogenic differentiation when cultured with fetal calf serum and dexamethasone, but the predominating cell type varies depending on conditions (Beresford et al., 1992). Specifically, when the steroid analog dexamethasone was present throughout the time course of culture, osteogenesis was favored; but when dexamethasone was present only during secondary culture, the adipogenetic pathway predominated as evidenced by lineage specific markers and cytological observation. Mouse derived CH3 10T1/2 cells are a multipotential cell line that, when treated with 5-azacytidine, undergoes terminal differentiation into adipocytes, myocytes and chondrocytes. The 5-azacytidine causes inhibition of DNA methylation and thus causes the activation of a few genes responsible for commitment to these lineages (Konieczny and Emerson, 1984).

In accordance with one aspect of the present invention, there is provided a composition and method for inducing human mesenchymal stem cells to preferentially differentiate into the adipogenic lineage, i.e., to differentiate into adipocytes.

Applicant has found that mesenchymal stem cells and in particular human mesenchymal stem cells (hMSC) can be directed to differentiate into adipocytes by treating the human mesenchymal stem cells with (i) a glucocorticoid and (ii) a compound which elevates intracellular cAMP levels by either upregulating cAMP production or by inhibiting degradation of cAMP; in particular a compound which inhibits compound(s) which degrade cAMP.

In a preferred aspect, the human mesenchymal stem cells are treated with a glucocorticoid and a compound which inhibits the activity of a compound which degrades cAMP; in particular a phosphodiesterase inhibitor. The cells are subsequently cultured in media containing insulin and fetal bovine serum.

Human mesenchymal stem cells, as well as their isolation and expansion, have been described in U.S. Pat. No. 5,486,359. As known in the art, human mesenchymal stem cells are capable of producing two or more different types (lineages) of mesenchymal cells or tissues and in particular connective tissue. The present invention provides a method for generating adipocytes from primary human mesenchymal stem cells (hMSCs) in a predictable and reproducible manner. The invention is unique in that it involves human cells in primary and passaged cultures rather than transformed or immortalized cell lines that are predetermined to enter the adipogenic pathway. hMSCs are capable of entering multiple lineages including the osteocytic, chondrocytic, myocytic, tendonocytic and stromogenic lineages and the present invention provides a method and composition for inducing hMSC's to differentiate

into adipocytes. In a preferred aspect, in accordance with the present invention, hMSC's are induced to differentiate into essentially only adipocytes, i.e., there is no essential production or commitment to cells of other mesenchymal lineages. The method may also be used for generating adipocytes from MSCs from other species such as rabbit, dog, rat and mouse.

The invention also provides methods to purify the adipocytes to obtain a highly purified population.

The method of the invention for the in vitro differentiation of human mesenchymal stem cells preferably derived from bone marrow into adipoblastic or adipocytic cells is useful to investigators wishing to study this developmental program in human cells in vitro. A better understanding of diseases of energy metabolism including obesity and obesity-related diabetes will also result from studies of the differentiation of mesenchymal stem cells to adipocytes. While a cellular and biochemical basis for obesity has long been suspected, advancements have been slow due to a lack of model systems with biochemical and molecular tools for study. Recent dramatic breakthroughs in the molecular basis of adipogenesis have opened new avenues towards understanding this pathway of mesenchymal cell differentiation, although a human model system such as the one described here has been lacking. The method will also have utility in the isolation and preparation of adipocytes for implantation into a patient for the purpose of tissue augmentation following trauma or cosmetic surgery.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B show that when human MSCs are treated in accordance with the invention, they undergo differentiation to the adipogenic lineage. FIG. 1A shows hMSCs (4.times.) cultured in normal hMSC media for the same period of time as FIG. 1B. There is no evidence of lipid containing vacuoles and the cells maintain the appearance of fibroblasts at high density. In FIG. 1B are hMSCs that were allowed to become confluent and then maintained in normal media for 10 days prior to adding the Adipogenic Induction media (containing methylisobutylxanthine and dexamethasone) for 48 hrs, and then changed to the insulin-containing adipocyte maintenance media for an additional 2 weeks. The lipid vacuoles are first apparent at about 5-7 days but increase in size and abundance over time.

FIG. 2A shows a similar control culture as FIG. 1A at higher magnification (20.times.). FIG. 2B show a culture of confluent hMSCs that were subjected to Adipogenic Induction media for 48 hours and then maintained in the Adipogenic Maintenance media for 14 days. The many lipid containing vacuoles of adipocytes are evident in a large proportion of the cells.

FIGS. 3A-3F show the results of culturing hMSCs under a
variety of conditions, only one of which shows a high degree
of adipogenic differentiation. All photos are at
10.times.magnification. FIG. 3A shows a culture of hMSCs
maintained in normal hMSC culture media alone. The cells grow
with a fibroblastic morphology. FIG. 3B shows a similar
culture that was treated with Adipogenic Induction media for
48 hours and then with Adipogenic Maintenance media for an
additional 14 days with media changes every 3 days. The
adipogenic cells, perhaps as many as 30-35% of the cells, are
evident as they contain the large refractile lipid vacuoles.
FIG. 3C shows a culture of hMSCs that were maintained in the
Adipogenic Maintenance media for 14 days but was never
subjected to the dexamethasone/methyl isobutylxanthine
treatment. The cells maintain a flat morphological appearance
with no evident vacuoles. FIG. 3D shows a culture of hMSCs
that were treated with normal hMSC media containing 1 .mu.M
dexamethasone for 48 hours and then cultured for 14 days in
the Adipogenic Maintenance media. The cells are disorganized
but show very few, if any, lipid vaculoes. FIG. 3E shows a
culture of hMSCs that was treated with normal hMSC media
containing 0.5 m. methyl-isobutylxanthine for 48 hours and
then was maintained for 14 days in the Adipogenic Maintenance
media. The cells retain a flat fibroblastic phenotype. FIG. 3F
shows a culture of hMSCs that was treated with a media that
induces the cells to differentiate along a osteogenic pathway.
This media contains 0.1 .mu.M dexamethasone, 10 mM
.beta.-glycerol phosphate and 50 .mu.M ascorbic acid
2-phosphate. The presence of refractile osteoid material is
evident but no large lipid vacuoles.

FIG. 4A shows a culture of hMSCs subjected to the Adipogenic
Induction media for 48 hours and then cultured for 14 days in
the Adipogenic Maintenance media. The large lipid vacuoles are
evident in this bright field image. The lipids can also be
revealed by using a fluorescent lipid soluble dye, such as
Nile Red, and viewing by epifluorescence illumination as shown
in FIG. 4B. Thus, the adipogenic cells can also be identified
using vital dyes and histological stains that label the lipid
vacuoles.

FIG. 5A shows hMSCs in culture which were not treated with
Adipogenic Induction media but which were cultured, fixed and
stained at the same time as the adipogenic cultures shown in
5B and 5C.

FIG. 5B shows hMSCs that were treated once for 48 hours with
Adipogenic Induction media and then cultured for an additional
three weeks in Adipogenic Maintenance media and then fixed in
neutral buffered formalin and stained with Oil Red O, a lipid
soluble dye that accumulates in the fat droplets.

FIG. 5C shows a dish of hMSCs that was retreated with fresh
Adipogenic Induction media for a second and third 48 hour
period to induce more hMSCs to become adipogenic. As many as
30-40% of the cells were converted to adipocytes by the three
induction treatments when viewed two weeks after the third

treatment. Panels 5A-5C were all stained with Oil Red O.

FIG. 6A shows the isolated adipogenic hMSCs that attached to the uppermost surface. The population is composed of greater than 99% adipogenic cells as evidenced by the lipid droplets in every cell.

FIG. 6B shows the non-adipogenic hMSCs that settled to the lower surface of the flask. Very few cells containing lipid droplets were present on the lower surface. These non-adipogenic cells could be treated with trypsin/EDTA and replated to another dish and be shown to retain adipogenic potential (data not shown), indicating that they remain as mesenchymal stem cells, capable of lineage progression.

DETAILED DESCRIPTION:

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, one aspect of the invention provides a composition which comprises an isolated, homogeneous population of human mesenchymal stem cells which have the potential to differentiate into cells of more than one mesenchymal tissue type, and a substance which induces cells from the mesenchymal stem cell population to differentiate into the adipogenic lineage.

In a preferred embodiment of this aspect of the invention mesenchymal stem cells are induced to differentiate into the adipogenic lineage by use of a glucocorticoid and a compound which either elevates intracellular levels of cAMP, for example, a cAMP analog or a compound which stimulates production of cAMP or inhibits degradation of cAMP; in particular a phosphodiesterase inhibitor.

Preferred examples of the glucocorticoid are selected from the group consisting of dexamethasone, hydrocortisone, cortisone, etc.

Preferred examples of the substance which elevate intracellular cAMP levels or are cAMP analogs include dibutyryl-cAMP, 8-CPT-cAMP (8-(4)-chlorophenylthio)-adenosine 3', 5' cyclic monophosphate; 8-bromo-cAMP; dioctanoyl-cAMP, Forskolin, etc.

Preferred examples of the substance which inhibits cAMP degradation by inhibiting the activity of phosphodiesterase is selected from the group consisting of methyl isobutylxanthine, theophylline; caffeine, and indomethacin.

The compound which elevates levels of cAMP and the glucocorticoid are used in amounts which are effective to induce hMSCs to differentiate into adipocytes. The cAMP regulating compound and glucocorticoid may be added to the hMSCs separately or in admixture with each other.

In general, the glucocorticoid is used in a concentration from about 0.1 to 10 micromolar, preferably from about 0.5 to 2 micromolar.

When employing a compound which inhibits degradation of cAMP, such a compound is generally employed in a concentration of about 0.1 to 10 millimolar and preferably from about 0.2 to 2 millimolar.

When employing a compound which upregulates cAMP production, such compound is generally employed in a concentration of from about 0.1 to 100 micromolar, preferably from about 0.5 to 10 micromolar.

It is to be understood that the above amounts are representative and the scope of the invention is not to be limited thereby.

Although one of the compounds which is employed to induce hMSCs to differentiate into adipocytes is one which regulates cAMP (either one which is known to upregulate cAMP production or one which prevents degradation of cAMP), the scope of the invention is not limited to any particular mechanism of action. Thus, for example, even though one of the compounds which may be used in the present invention is a phosphodiesterase inhibitor which is known to inhibit degradation of cAMP by inhibiting phosphodiesterase degradation of cAMP, the invention is not limited to a mechanism of action which is dependent upon preventing degradation of cAMP. Thus, for example, the phosphodiesterase inhibitor may be effective for inducing differentiation of hMSCs to adipocytes by a mechanism of action other than inhibiting degradation of cAMP.

Compounds in addition to (i) a glucocorticoid and (ii) a cAMP regulator may be used for inducing hMSCs to differentiate into adipocytes. Thus, for example, in a preferred embodiment insulin is also employed in conjunction with the cAMP regulator and glucocorticoid.

In a preferred embodiment, there is provided a composition for inducing hMSCs to differentiate into adipocytes which is comprised of (i) a glucocorticoid, (ii) a compound which regulates cAMP and in particular a compound which inhibits cAMP degradation such as, a phosphodiesterase inhibitor, (iii) insulin or insulin-like growth factor and (iv) glucose.

The addition of compounds as hereinabove described to induce differentiation of hMSCs to adipocytes in accordance with the invention does not require that all of the treated hMSCs be induced to differentiate into adipocytes. Thus, in accordance with an aspect of the present invention there is produced a composition comprised of human mesenchymal stem cells and adipocytes wherein based on the two components the adipocytes are present in an amount of at least 5 wt. % and preferably at least 15 wt. %. The amount of adipocytes may be up to 50 wt. % or higher, based on the two components.

In accordance with a preferred embodiment, the composition which is generated is essentially free of committed cells of the mesenchymal lineage other than adipocytes. In a particular preferred embodiment, there are less than one percent of committed cells of the mesenchymal lineage other than adipocytes, and more preferably, less than 0.1% and most preferably no committed cells of the mesenchymal lineage other than adipocytes.

Although treatment of hMSCs in accordance with the invention produces a mixture of adipocytes and undifferentiated hMSCs, the produced adipocytes may be recovered from the mixture to produce an isolated population of adipocytes. Representative procedures for recovering adipocytes are described in the examples which form a part of this application.

In accordance with an aspect of the present invention, hMSCs may be treated to induce differentiation into adipocytes in a manner such that such differentiation is effected in vitro or in vivo.

Thus, for example, hMSCs may be admixed with compounds as hereinabove described which induce differentiation into hMSCs and the resulting mixture employed in vivo to induce differentiation to adipocytes in vivo. Thus, for example, the mixture without culturing in vitro for a period of time to induce differentiation in vitro may be employed in a suitable matrix (for example of the type hereinafter described) to induce differentiation of the hMSCs to adipocytes in vivo.

Thus, in accordance with an aspect of the present invention there is provided a composition comprised of human mesenchymal stem cells, a glucocorticoid and a cAMP regulator (a compound(s) which upregulates cAMP production or inhibits cAMP degradation). Such a composition may be employed to produce adipocytes in vitro or may be employed to induce differentiation of hMSCs in vivo.

The ability to generate large percentages of adipogenic cells from a population of hMSCs will allow greater numbers of cells for implantation or research studies. Fewer hMSCs would be needed as starting material. By repeating the adipogenic induction step more times, it should be possible to induce most of the hMSCs in a population to adipocytes. In the case where there is a mixture of cells, adipogenic hMSCs can easily be isolated by their buoyant density. The isolation of a highly enriched population of adipocytes from cultured hMSCs will also allow for a detailed characterization of the adipocyte phenotype.

The adipocytes can be used with a variety of materials to form a composition for purposes such as reconstructive surgery. The cells may be combined with a biomatrix to form a two dimensional or three dimensional material as needed. Surgeons routinely use fat pads and fatty tissues from remote sites to build up an area where tissue has been removed. This often

involves a separate procedure with its inherent risks. As an
alternative, hMSCs could be isolated from the patient and
grown in culture. The hMSCs could then be mixed with a
biocompatible material such as collagen, collagen sponge,
alginate, polylactic acid material etc. to form a composite.
The composite would then be treated to induce adipogenic
differentiation of the MSCs in vitro for 1-3 weeks, then
implanted when needed. For example, adipogenic MSCs could be
mixed with a solubilized collagen or other biomaterial which
is then allowed to gel to form a three dimensional composite
that could be used for breast augmentation following
mastectomy. Such a composite could be formed or sculpted to
the appropriate size and shape. Another composition includes
the culturing of hMSCs on the acellular skin matrix that is
currently on the market such as the product by LifeCell
Corporation. In this format the cells would be cultured to
populate the matrix and then caused to differentiate as
described. The matrix with the adipogenic cells could then be
cut by the surgeon to fit the site of reconstruction. As an
alternative hMSCs could be induced to become adipocytes prior
to their introduction into the biocompatible materials. As
another alternative, hMSCs in combination with compounds which
promote differentiation into adipocytes may be used with a
biomatrix as described without culturing for a period of time
to induce differentiation whereby differentiation is induced
in whole or in part in vivo.

Similar to their use in reconstructive surgery, adipogenic
hMSCs will be of use in elective cosmetic surgery in much the
same way; to build up underlying tissue below the skin with a
composite of autologous cells and biocompatible material.

The invention will be further described with respect to the
following examples; however, the scope of the invention is not
to be limited thereby. Unless otherwise described percentages
and parts are by weight.

Biochemical Markers of Adipocytes

A number of molecules that are specific markers of adipocytes
have been described in the literature that will be useful to
characterize the adipocytes derived from hMSCs. These include
enzymes involved in the interconversion of fatty acids to
triglycerides such as stearoyl-CoA-desaturase (SCDI) or the
insulin responsive glucose transporter (GLUT4). The product of
the ob gene, leptin is a 16,000 molecular weight polypeptide
that is only expressed in pre-adipose cells or adipose tissue.
The expression of CCAAT enhancer binding protein, C/EBP, has
been shown to precede the expression of several markers of
adipogenic differentiation and it is thought to play a key
role in adipocyte development. Another marker is 422 adipose
P2 (422/aP2), a protein whose expression is enhanced during
adipocyte differentiation (Cheneval, et al, 1991.). This
differentiation pathway is thought also to involve peroxisome
proliferation-activated receptor .gamma.2 (PPAR .gamma.2),
which is involved in the initiation of transcription of
adipocyte genes (Tontonoz, et al, 1994). Studies using these

markers and the described methods will allow a more detailed
analysis of the lineage progression of mesenchymal stem cell
to adipocyte differentiation.

Lipid soluble dyes as markers of adipocyte differentiation

Lipid soluble dyes are available to stain lipid vaculoes in
adipocytes. These include Nile Red, Nile Blue, Sudan Black and
Oil Red O, among others. Each of these hydrophobic dyes has a
propensity to accumulate in the lipid containing vaculoes of
the developing adipocytes and can readily identify the
adipogenic cells in populations of differentiating MSCS. At
least one of these dyes can be used to isolate adipocytes from
non-differentiated cells using a fluorescence activated cell
sorter (FACS). An example of the use of Nile Red to identify
adipogenic hMSCs is shown in FIG. 4.

EXAMPLE 1

Generation of Adipocytes from Human MSCs

Human MSCs are isolated from the red bone marrow of volunteer
donors as described in U.S. Pat. No. 5,486,359.

The cells are grown until colonies are well established and at
this point the cells are subcultured (1 to 3) or they can be
taken to assay for in vitro adipogenesis. For the adipogenesis
assay, hMSCs are subcultured into 35 mm tissue culture dishes
at 100,000 cells per dish and fed with 2 milliliters normal
hMSC Media (Dulbecco's Modified Eagle's Media (DMEM), 10%
selected Fetal Bovine Serum (FBS) and antibiotic/antimycotic
mixture (1.times.) (Life Technologies, Inc.)) and cells are
maintained at 37.degree. C., 5% CO.sub.2 and 90% humidity. The
cells are refed with the fresh media every third day and are
allowed to multiply and become confluent. The cells are
maintained after reaching confluence by refeeding every third
day and this time period of post confluence culturing enhances
the adipogenic response in the next step (at least out to 14
days). The differentiation into adipocytes is initiated by
changing the media to 2 ml of Adipogenic Induction Media (DMEM
with 10% fetal bovine serum containing 10 .mu.g/ml insulin
(human recombinant, Boehringer Mannheim Corp.), 0.5 mM methyl
isobutylxanthine (MIX)(Sigma Chemical Co.), 1 uM dexamethasone
(Dex)(Sigma Chemical Co.)). This media is left on the cells
for 48 hrs with cells maintained at 37.degree. C., 5%
CO.sub.2, 90% humidity and is then replaced with Adipogenic
Maintenance Media (DMEM containing 10% FBS and 10 .mu.g/ml
insulin). The medium is changed every 3-4 days. The hMSCs
begin to show small lipid vacuoles in 3-7 days and these
enlarge and become more numerous over time, out to at least 30
days. There are several variations that have been successfully
tried.

When human MSCs are treated as described above, they undergo
differentiation to the adipogenic lineage, as shown in FIG. 1.
FIG. 1A shows hMSCs (4.times.) cultured in normal hMSC media
for the same period of time as FIG. 1B. There is no evidence

of lipid containing vacuoles and the cells maintain the appearance of fibroblasts at high density. In FIG. 1B are hMSCs that were allowed to become confluent and then maintained for 10 days prior to adding the Adipogenic Induction Media for 48 hrs, and then changed to the Adipogenic Maintenance Media for an additional 2 weeks. The lipid vacuoles are first apparent at about 3-7 days but increase in size and abundance over time. FIG. 2A shows a similar control culture as FIG. 1A at higher magnification (20.times.). FIG. 2B show a culture of confluent hMSCs that were subjected to Adipogenic Induction Media for 48 hours and then maintained in Adipogenic Maintenance Media for 14 days. The many lipid containing vacuoles of adipocytes are evident in a large proportion of the cells.

FIG. 3 shows the results of culturing hMSCs under a variety of conditions, only one of which shows a high degree of adipogenic differentiation. All photos are at 10.times.magnification. FIG. 3A shows a culture of hMSCs maintained in normal hMSC culture media with no additives. The cells grow with a fibroblastic morphology. FIG. 3B shows a similar culture that was treated with Adipogenic Induction Media for 48 hours and then with Adipogenic Maintenance Media for an additional 14 days with media changes every 3 days. The adipogenic cells, perhaps as many as 30-35% of the cells, are evident as they contain the large refractile lipid vacuoles. FIG. 3C shows a culture of hMSCs that were maintained in the Adipogenic Maintenance Media for 14 days but was never subjected to the dexamethasone/methyl isobutylxanthine treatment. The cells maintain a flat morphological appearance with no evident vacuoles. FIG. 3D shows a culture of hMSCs that were treated with normal hMSC media containing 1 .mu.M dexamethasone for 48 hours and then cultured for 14 days in the Adipogenic Maintenance Media. The cells are disorganized but show very few, if any, lipid vacuoles. FIG. 3E shows a culture of hMSCs that was treated with normal hMSC containing 0.5 ml. methyl isobutylxanthine during the induction period and then was maintained for 14 days in the Adipogenic Maintenance Media. The cells retain a flat fibroblastic phenotype. FIG. 3F shows a culture of hMSCs that was treated with a media that induces the cells along a osteogenic pathway. This media contains 0.1 .mu.M dexamethasone, 10 mM .mu.-glycerol phosphate and 50 .mu.M ascorbic acid 2-phosphate. The presence of refractile osteoid material is evident but no large lipid vacuoles.

The adipogenic cells can also be identified using vital dyes and histological stains that label the lipid vacuoles. FIG. 4A shows a culture of hMSCs subjected to the adipogenic treatment and cultured for 14 days in the Adipogenic Maintenance Media. The large lipid vacuoles are evident in this bright field image. But the lipids can also be revealed by using a fluorescent lipid soluble dye such as Nile Red (Greenspan, et al. 1985) and viewing by epifluorescence illumination as shown in FIG. 4B.

The results shown here have been reproduced several times with

hMSCs derived from different donors and additional information on the method is described here. Similar results have been obtained with hMSCs from all individuals tested (4 or more donors). The percentage of cells that become lipid containing adipocytes varies depending on the specifics of culturing. Specifically, those cells that were allowed to become completely confluent and maintained this way for up to 2 weeks prior to adipocyte induction, showed a much higher percentage of adipocytes overtime than cultures that were induced prior to confluence or at confluence. As many as 30-35% of the hMSCs appear adipogenic at 2 weeks post induction when treated as described herein as shown in FIG. 1B. hMSCs can be cultured in various sizes of culture ware with equal success so obtaining larger numbers of cells should not present any problem.

EXAMPLE 2

Enhancing Adipogenic Differentiation

These experiments were performed to determine whether a population of human mesenchymal stem cells growing in culture can be treated to induce adipogenic differentiation (which induces a percentage of 5-10% of the cells to become adipocytes), and then retreated at a later time to induce more of the hMSCs to differentiate. Experiments were also performed to examine whether it is possible to purify a population of induced adipogenic cells from the mixed culture of hMSCs and adipogenic MSCs that result from the treatment with adipogenic agents. Both sets of experiments were successful as described below and in the accompanying figures.

hMSCs were induced to the adipogenic phenotype by the culturing in Adipogenic Induction Media for 48 hours as described. The media was then changed to Adipogenic Maintenance Media and cells were cultured at 37.degree. C. in a 5% CO.sub.2 atmosphere for 3 to 6 days until there were noticeable lipid droplets visible within cells. Approximately 5-10% of the cells became adipogenic as seen in FIG. 5. FIG. 5A shows hMSCs in culture which were not treated with either adipogenic medium but which were cultured, fixed and stained at the same time as the adipogenic cultures shown in 5B and 5C. FIG. 5B was treated once with Adipogenic Induction Media and then cultured in Adipogenic Maintenance Media for an additional three weeks and then fixed in neutral buffered formalin and stained with Oil Red O, a lipid soluble dye that accumulates in the fat droplets. A dish of hMSCs was also retreated with fresh Adipogenic Induction Media for a second and third 48 hour period to induce more hMSCs to become adipogenic as shown in FIG. 5C. As many as 30-40% of the cells were converted to adipocytes by the three induction treatments when viewed two weeks after the third treatment. Panels 5A-5C were all stained with Oil Red O.

EXAMPLE 3

Isolation of Adipocytes from hMSCs

The generation of adipocytes from human mesenchymal stem cells by the conditions described above produces large numbers of adipocytes, perhaps as many as 30%-40% of the cells present. For uses requiring a pure population, the adipocytes can be isolated from the non-adipogenic hMSCs by several methods as listed below.

Method one for isolating adipogenic hMSCs uses density gradient centrifugation and takes advantage of the greater buoyancy of the lipid-containing adipogenic cells. In this method, cultures containing hMSCs and adipocytes derived from hMSCs are treated with 0.05% trypsin/0.53 mM EDTA to remove the cells from the culture dish and the cells are washed by adding 10 ml of normal hMSC media and centrifuged for 10 minutes at 1000 rpm in the GS-6R centrifuge (Beckman Instruments, Inc.) at 20.degree. C. The pelleted cells containing adipocytes and hMSCs are resuspended in 2 ml of the Adipogenic Maintenance Media and carefully layered on top of 8 ml of Percoll of a density of 1.045 g/ml. The tubes are centrifuged at 2,200 rpm (1100.times.g) in a Beckman GS-6R centrifuge for 20 minutes at 3.degree. C. The adipocytes are recovered in the uppermost 2 mls and at the interface with the 1.045 density Percoll. (The non-adipogenic MSCs enter into the 1.045 density Percoll and can be recovered at the bottom of the tube.) The recovered adipocytes are washed by addition of 10 mls of the Adipogenic Maintenance Media and centrifuged at 1000 rpm for 10 min at 20.degree. C. in the GS-6R centrifuge. The adipocytes are replated at a density of 150,000 cells per 35 mm dish in Adipogenic Maintenance Media and returned to the incubator.

Method two for isolating adipogenic hMSCs uses fluorescence activated cell sorting (FACS). The hMSCs differentiating into adipocytes in a culture can be isolated by using a lipid soluble fluorescent dye, such as Nile Red (10-100 ug/ml) to stain lipid vacuole-containing adipocytes. The culture is then treated with trypsin/EDTA as above to release the cells from the culture vessel and subjecting the mixed population to fluorescence activated cell sorting (FACS). The parameters on the machine are adjusted to select and recover adipogenic cells in the population and they can be used directly or replated and cultured in the incubator.

As described below, Method three of isolating the adipogenic cells in a mixed population is to trypsin/EDTA treat and wash the cells as above. The cells are then placed in a tissue culture flask and the flask is filled with media. The flask is closed tightly and turned upside-down so that the surface treated for cell adhesion is uppermost. The buoyant, lipid-droplet-containing adipocytes rise to top and attach to the surface of the flask. The next day the media is removed and the flask rinsed with fresh media and the flask turned right-side-up. The flask, now with only enough media to cover the cell layer, is returned to the incubator for further maintenance.

Adipogenic hMSCs accumulate lipid droplets which decreases the

bouyant density of the cells. The adipogenic hMSCs were then
isolated as follows. The dish of cells containing adipocytes
and non-adipogenic hMSCs from a culture that were treated for
only one 48 hr induction period and then grown for three
weeks, was treated with 0.05% trypsin and 5 mM EDTA for 3-5
minutes to release the cells from the dish. The cells were
then rinsed from the dish with 10 ml of fresh media and placed
in a 25 cm.sup.2 flask and the flask was filled to the brim
with fresh media. The flask was turned upside down so the
usual culture surface was uppermost and the flask placed in
the 37.degree. C. incubator overnight. The more bouyant
adipogenic cells rose to the top and attached to the surface,
while the non-adipogenic hMSCs settled to the bottom surface
and attached. The following day photos were taken of the cells
on each surface as shown in FIG. 6. FIG. 6a shows the
adipogenic hMSCs that attached to the uppermost surface. The
population is composed of greater than 99% adipogenic cells as
evidenced by the lipid droplets in every cell. FIG. 6b shows
the non-adipogenic hMSCs that settled to the lower surface of
the flask. Very few cells containing lipid droplets were
present on the lower surface. These non-adipogenic cells could
be treated with trypsin/EDTA and replated to another dish and
be shown to retain adipogenic potential (data not shown),
indicating that they remain as mesenchymal stem cells, capable
of lineage progression.

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CLAIMS:

What is claimed is:

1. A composition which comprises mesenchymal stem cells which have been isolated from a human and which have potential to differentiate into cells of more than one connective tissue type and a substance in an amount effective to induce said stem cells to differentiate into the adipogenic lineage.

2. The composition of claim 1 wherein the substance which induces cells from the mesenchymal stem cell population to differentiate into the adipogenic lineage comprises a glucocorticoid and a compound which elevates intracellular levels of cAMP.

3. The composition of claim 2 wherein the glucocorticoid is selected from the group consisting of dexamethasone, hydrocortisone, and cortisone.

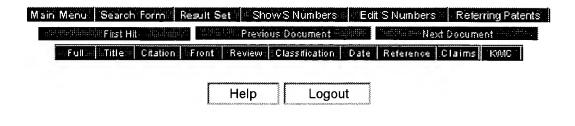4. The composition of claim 2 wherein the substance which

elevates intracellular cAMP is selected from the group
consisting of dibutyryl-cAMP, 8-CPT-cAMP
(8-(4)chlorophenylthio)-adenosine 3', 5' cyclic monophosphate;
8-bromo-cAMP; dioctanoyl-cAMP and Forskolin.

5. The composition of claim 2 wherein the substance which
induces cells from the mesenchymal stem cell population to
differentiate into the adipogenic lineage further comprises
insulin.

6. A process for inducing human mesenchymal stem cells to
differentiate into adipocytes, comprising:

contacting human mesenchymal stem cells with (i) a
glucocorticoid and (ii) a compound which elevates
intracellular levels of cAMP, said glucocorticoid and said
compound which elevates intracellular levels of cAMP being
employed in an amount sufficient to induce human mesenchymal
stem cells to differentiate into adipocytes.

7. The process of claim 6 which further comprises isolating
the adipocytes from remaining hMSCs.

8. The composition of claim 1 wherein the substance which
induces cells from the mesenchymal stem cell population to
differentiate into the adipogenic lineage comprises a
glucocorticoid and a compound which inhibits degradation of
cAMP.

9. The composition of claim 8 wherein the substance which
inhibits degradation of cAMP is a phosphodiesterase inhibitor
selected from the group consisting of methyl isobutylxanthine,
theophylline, caffeine, and indomethacin.

10. The composition of claim 8 wherein the substance which
induces cells from the mesenchymal stem cell population to
differentiate into the adipogenic lineage further comprises
insulin.

11. The process of claim 10 which further comprises isolating
the adipocytes from remaining hMSCs.

12. The process of claim 10 wherein (ii) is a
phosphodiesterase inhibitor.

13. The process of claim 12 wherein (i) and (ii) are in
admixture with each other.

14. A process for inducing human mesenchymal stem cells to
differentiate into adipocytes, comprising:

contacting human mesenchymal stem cells with (i) a
glucocorticoid and (ii) a compound which inhibits degradation
of cAMP, said glucocorticoid and said compound which inhibits
degradation of cAMP being employed in an amount sufficient to
induce human mesenchymal stem cells to differentiate into
adipocytes.

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Document Number 1

    Entry 1 of 1   File: USPT   Jan 7, 1997

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INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Gerson; Stanton L.	Pepper Pike	OH	N/A	N/A
Caplan; Arnold I.	Cleveland Heights	OH	N/A	N/A
Haynesworth; Stephen E.	Cleveland Heights	OH	N/A	N/A

ASSIGNEE INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Case Western Reserve University	Cleveland	OH	N/A	N/A	02

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PRIMARY-EXAMINER: Fleisher; Mindy

ASSISTANT-EXAMINER: Railey, II; Johnny F.

ATTY-AGENT-FIRM: Herron; Charles J. Olstein; Elliot M.

ABSTRACT:

Genetically engineered human stem cells that carry within them
genes of interest particularly for the expression of
physiologically or pharmacologically active proteins or for
use in gene therapy. In addition to correction of genetic
disorders, is the ability to introduce, in a targeted manner,
additional copies of essential genes to allow expression in
proliferating, nondifferentiating cells of certain gene
products. These genes can be, for example, hormones matrix
proteins, cytokines, adhesion molecules, detoxification
enzymes and "rebuilding" proteins important in tissue repair.
21 Claims, 11 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 7

BRIEF SUMMARY:

The present invention is directed to a totally unexplored
application of human stem cells, i.e. that of genetically
engineered cell that carry within them genes of interest
particularly for the expression of physiologically or
pharmacologically active proteins or for use in gene therapy.

In accordance with the present invention it has been
discovered that human mesenchymal stem cells (MSCs) or human
mesenchymal progenitor cells can be used as host cells for the
expression of exogenous gene products. One aspect of the
invention relates to the discovery and development of the
technology to isolate these cells, mitotically proliferate
them in cell culture and introduce them back in vivo into the
same recipient. These culture-expanded cells home back to the
marrow and enhance hematopoietic recovery in the marrow
transplant setting. Furthermore, these cells can be
manipulated for cellular therapy, e.g. expanded, purified,
selected and maintained for clinical use while still
mantaining their precursor phenotype. Part of this

manipulation is the characterization of such cells and their cryopreservation for future use.

Mesenchymal stem cells (MSCs) can be derived from marrow, periosteum, dermis and other tissues of mesodermal origin. They are the formative pluripotential blast cells that differentiate into the specific types of connective tissues (i.e. the tissues of the body that support the specialized elements; particularly adipose, areolar, osseous, cartilaginous, elastic, marrow stroma, muscle, and fibrous connective tissues) depending upon various in vivo or in vitro environmental influences. Although these cells are normally present at very low frequencies in bone marrow, the inventors of the present invention have discovered a process for isolating, purifying, and greatly replicating the marrow-derived mesenchymal stems cells in culture, i.e. in vitro. This discovery is the subject of U.S. patents and co-pending applications, for example, Caplan and Haynesworth, U.S. Pat. Nos. 5,197,985 and 5,226,914 and PCT Publication No. WO 92/22584 (published 23 Dec. 1992) as well as numerous literature references by Caplan and Haynesworth.

Isolated human hematopoietic stem cells have also been described, for example, in Tsuksamoto et al., U.S. Pat. No. 5,061,620 (October 1991) and reviewed in Edgington, Biotechnology, 10:1099-1106 (1992) and the references cited therein. These are distinguished from MSCs by their ability to differentiate into myeloid and lymphoid blood cells.

In its principal embodiment the invention relates to isolated human mesenchymal stem cells capable of expressing incorporated genetic material of interest. Human stem cells are obtained from the individual donor and rendered substantially isolated from other cells and constituitive donor proteins and other components. It is contemplated that the transformed cells and the expression products of the incorporated genetic material can be used alone or in combination with other cells and/or compositions.

Another aspect of the invention relates to the development of the technology to introduce foreign genes into these progenitor cell cultures, such that all progeny of the cells carry the new genetic material. In addition, cell delivery of the transformed cells is an important component of the process and includes infusion and direct depot injection into periosteal, bone marrow, muscle and subcutaneous sites.

By virtue of the present invention, genes can be introduced into cells which are then returned to the autologous or syngeneic donor where gene expression will effect its therapeutic benefit. Examples of such applications include genes which have a central role in mesenchymal cell maintenance, tissue development, remodeling, repair and in vivo production of extracellular gene products. One example is the gene for normal type I collagen, which can be introduced into the MSCs of osteogenesis imperfecta patients who have a defect in collagen type I.

In addition to the correction of genetic disorders, a unique potential of this technology is the ability to introduce, in a targeted manner, additional copies of essential genes to allow augmented expression of certain gene products. These genes can be, for example, hormones, matrix proteins, cell membrane proteins cytokines, adhesion molecules, detoxification enzymes and "rebuilding" proteins important in tissue repair.

An additional application is the use of introduced genes to alter the phenotype of mesenchymal stem cells and their differentiated progeny for specific therapeutic applications. This includes intracellular gene products, signal transduction molecules, cell surface proteins, extracellular gene expression products and hormone receptors. Disease states and procedures for which such treatments have application include genetic disorders of the musculoskeletal system, diseases of bone and cartilage, the bone marrow, inflammatory conditions, muscle degenerative diseases, malignancies and autologous or allogeneic bone or bone marrow transplantation.

In one embodiment, the isolated human stem cells are preferably mesenchymal stem cells that have been transformed with at least one DNA sequence capable of expressing those translation products capable of packaging a viral sequence so as to be gene therapy producer cells. In a preferred embodiment of this aspect, the isolated human stem cells have been transformed with a DNA sequence comprising a retroviral 5' LTR and, under the transcriptional control thereof, at least one of a retroviral gag, pol or env gene. In another aspect, the isolated human stem cells have also been transformed with a DNA sequence comprising a retroviral packaging signal sequence and incorporated genetic material to be expressed under the control of a promoter therefor so as to be incompetent retroviruses. Also contemplated is the transfection of MSCs or committed stromoblasts to initiate, modulate or augment hematopoiesis.

An additional use of these cells is as gene-transduced producer cells. Defective retrovirus delivery vectors can be inserted into stem cells which thereby become a source of retroviral vectors which, when cocultured in vitro or injected in vivo, deliver genes of interest to targeted areas in an ongoing fashion thereby making it possible to use autologous human cells as producer cells. Thus, the stem cells provide a self-renewing supply of cells carrying the new gene or genes. These cells can be prepared in vitro and introduced one or more times in vivo. Therapy can be initiated by extracting a small number of cells initially and repeated as needed using culture-expanded and even cryopreserved cells. In this way, for example, the patient with osteogenesis imperfecta will slowly and autonomously repopulate skeletal tissue with recombinant type I collagen as the patient's bone grows and remodels.

Virtually all genetic lesions of mesenchymal cells or tissue can be treated or "corrected" by this technology. A key

component is the ability to deliver these gene-carrying stem cells to the proper tissue under the conditions that the stem cells will expand and repopulate the tissue space. Patient preparation for introduction of mesenchymal stem cells includes, but is not limited to, (a) marrow ablation by chemotherapy and/or irradiation in conjunction with marrow transplantation, (b) bone or cartilage reconstruction; (c) immunosuppression in the setting of allogeneic cell therapy; and (d) direct tissue infiltration of "corrected" cells without preparation, particularly where the transduced cells might have a survival advantage, an advantage during differentiation or an advantage in function (such as might be the case when correcting a muscle disorder such as muscular dystrophy with the dystrophin or similar gene). An additional application is in the tagging of MSCs prepared for use in vivo alone or as applied to any indwelling device, such as, for example, an orthopedic device in which it is of interest to "mark" the MSC's and observe their survival, maintenance and differentiation and their association with the device over time.

The advantages provided by the present invention include (a) the ability to culturally expand human stem cells for (re)infusion where they will localize to mesenchymal tissue spaces; (b) the ability to culturally expand and cryopreserve human mesenchymal stem cells which can be used as hosts for stable, heritable gene transfer; (c) the ability to recover genetically altered cells after installation in vivo; (d) the ability to match a genetic therapy to a wide variety of disorders, pinpointing the genetic alteration to the target tissue; and (e) the ability of newly introduced genes within human stem cells and their progeny to be expressed in a less restrictive fashion than other cells, thereby expanding the potential application in treating medical disease.

FIG. 1. Construction of vM5neolacZ: LacZ was cloned into a unique BamHl site of pM5neo. LacZ and neo are transcribed from the MPSV 5' LTR. This is described in detail in Example 1.

FIG. 2. LacZ expression as a function of retroviral infection: MSCs were infected with vM5neolacZ retroviral supernatant and Polybrene either on one day or three consecutive days. Cells were then plated at low density to allow clusters to form and stained with X-gal. Three daily infections of MSCs increased the efficiency of lacZ expressing colonies 1.5-2.5 fold. This is described in detail in Example 2.

FIG. 3. G418 survival curve and LacZ expression following retroviral transduction: Uninfected MSCs and MSCs retrovirally transduced with vM5neolacZ were plated at $5.times.10.sup.4$ cells/ml in 12 well plates and selected in 250-1000 .mu.g/ml G418, a neomycin analog (20), for four weeks. Selected colonies were stained with X-gal and counterstained with crystal violet. MSCs infected with vM5neolacZ are G418 resistant and express lacZ at a high frequency. The number of lacZ.sup.+ colonies approaches 100% with increased G418 concentration.

FIG. 4. LacZ expression in MSCs In-vitro: MSCs were retrovirally transduced with vM5neolacZ, plated at 5.times.10.sup.4 cells/ml with or without G418 for three weeks, stained with X-gal, which stains LacZ.sup.+ cells blue, and counterstained with crystal violet which stains the nucleus purple. Colonies shown were selected in G418.

FIGS. 5A-5G. LacZ expression in MSCs and osteocytes following in-vivo growth in ceramic cubes: Untransduced and vMneolacZ transduced MSCs and transduced NIH 3T3 cells were loaded into ceramic cubes and implanted subcutaneously into SCID mice. After six weeks the cubes were removed, fixed and stained for lacZ expression and .beta.-galactosidase product with X-gal. The cubes were then demineralized, embedded in paraffin, sectioned and counterstained with neutral red (A,C,E,F) or Mallory Heidenhain (B,D). The Legend for FIG. 5 is: B bone; C, ceramic; Ob, osteoblast; Oc, osteocyte. The results show that:

A,B) Untransduced MSCs form bone within the ceramics but show no lacZ.sup.+ cells.

C-F) Transduced MSCs form bone within the ceramics and lacZ.sup.+ (blue) osteocytes within bony lacunae can be seen.

G) Transduced NIH 3T3 cells are lacZ.sup.+ within the cube but fail to show bone formation.

The following detailed description, including the definitions that follow below, will aid in a fuller understanding and exemplification of the invention.

As used herein "substantially homologous," which can refer both to nucleic acid and amino acid sequences, means that a particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences. For purposes of the present invention, sequences having greater than 90 percent homology, equivalent biological activity, and equivalent expression characteristics are considered substantially homologous. For purposes of determining homology, truncation of the mature sequence should be disregarded. Sequences having lesser degrees of homology, comparable bioactivity, and equivalent expression characteristics are considered equivalents.

As used herein "DNA segment" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, which has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the segment and its component nucleotide sequences by standard biochemical methods, for example, using a cloning vector. Such segments are provided in the form of an open reading frame

uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Sequences of non-translated DNA may be present downstream from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions.

As used herein "nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. Generally, DNA segments encoding the proteins provided by this invention are assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon.

As used herein "recombinant expression vector" refers to a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

As used herein "recombinant expression system" means a substantially homogeneous monoculture of human mesenchymal stem cells which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. In the present case the human mesenchymal stem cells constituting the system are the progeny of a single ancestral transformant. Recombinant expression systems as defined herein will express heterologous protein upon induction of the regulatory elements linked to the DNA segment or synthetic gene to be expressed.

A preferred aspect the invention contemplates the use of human mesenchymal stem cells that include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH2, deposited with the ATCC under accession number HB10743. The human mesenchymal stem cells can further include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH3, deposited with the ATCC under accession number HB10744. The mesenchymal stem cells further include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH4, deposited with the ATCC under accession number HB10745.

In another aspect, the invention contemplates a composition that comprises the transformed mesenchymal stem cells in a medium that stimulates their culture expansion but does not

stimulate their differentiation. Preferably the medium
comprises a supplemented DMEM particularly wherein the medium
includes fetal bovine (e.g. calf) serum. The composition can
also be supplemented with an antibiotic and antimycotic
composition. In other embodiments of this aspect, the
composition comprises the stem cells in supplement BGJ.sub.b
medium or supplemented F-12 Nutrient Mixture.

In another aspect of the invention, the stem cells are
isolated from other cells by density gradient fractionation,
such as by Percoll gradient fractionation. The isolated stem
cells are preferably transformed with at least one DNA
sequence capable of expressing those translation products
capable of packaging a viral sequence.

The structure and life cycle of retroviruses makes them
ideally suited to be gene-transfer vehicles since (i) the
majority of sequences coding for their structural genes are
deleted and replaced by the gene(s) of interest which are
transcribed under control of the retroviral regulatory
sequences within its long terminal repeat (LTR) region and
(ii) they replicate through a DNA intermediate that integrates
into the host genome. Although the sites of integration appear
to be random with respect to the host genome, the provirus
integrates with a defined structure in low copy number. Most
of the viral gene sequences can function when supplied in
trans. Generally regarding retroviral mediated gene transfer,
see McLachlin et al., Progress in Nucleic Acid Research and
Molecular Biology, 38:91-135 (1990).

Retroviruses are RNA viruses; that is, the viral genome is
RNA. This genomic RNA is, however, reverse transcribed into a
DNA intermediate which is integrated very efficiently into the
chromosomal DNA of infected cells. This integrated DNA
intermediate is referred to as a provirus. As shown in FIG. 1,
the retroviral genome and the proviral DNA have three genes:
the gag, the pol and the env, which are flanked by two long
terminal repeat (LTR) sequences. The gag gene encodes the
internal structural (nucleocapsid) proteins, the pol gene
encodes the RNA-directed DNA polymerase (reverse
transcriptase); and the env gene encodes viral envelope
glycoproteins. The 5' and 3' LTRs serve to promote
transcription and polyadenylation of virion RNAs.

Adjacent to the 5' LTR are sequences necessary for reverse
transcription of the genome (the tRNA primer binding site) and
for efficient encapsidation of viral RNA into particles (the
Psi site). Mulligan, R. C., In: Experimental Manipulation of
Gene Expression, M. Inouye (ed). Proceedings of the National
Academy of Sciences, U.S.A. 81:6349-6353 (1984).

In order to generate a viral particle containing the
recombinant genome, it is necessary to develop cell lines that
provide packaging "help". To accomplish this, a plasmid(s),
encoding, for example, the retroviral structural genes gag,
pol, and env, is introduced into an otherwise untransformed
tissue cell line by conventional calcium-phosphate-mediated

DNA transfection, Wigler, et al., Cell 11:223 (1977). This plasmid-containing cells are referred to as a "packaging cell line." These plasmid containing packaging cell lines can be maintained as such or a replication incompetent retroviral vector can be introduced into the cell's genome. In the latter case, the genomic RNA generated by the vector construct combines with the constitutively expressed retroviral structural proteins of the packaging line, resulting in the release of retroviral particles into the culture medium. A stable cell line containing the structural gene sequences of the retroviruses is a retroviral "producer cell line."

Because genes can be introduced into progenitor cells using a retroviral vector, they can be "on" (subject to) the retroviral vector control; in such a case, the gene of interest is transcribed from a retroviral promoter. A promoter is a specific nucleotide sequence recognized by RNA polymerase molecules that start RNA synthesis. Alternatively, retroviral vectors having additional promoter elements (in addition to the promoter incorporated in the recombinant retrovirus) which are responsible for the transcription of the genetic material of interest, can be used. For example, a construct in which there is an additional promoter modulated by an external factor or cue can be used, making it possible to control the level of polypeptides being produced by the progenitor cells by activating that external factor of cue. For example, heat shock proteins are proteins encoded by genes in which the promoter is regulated by temperature. The promoter of the gene which encodes the metal-containing protein metallothionine is responsive to cadmium (Cd^{++}) ions. Incorporation of this promoter or another promoter influenced by external cues also makes it possible to regulate the production of the polypeptide by the engineered progenitor cells.

It is also possible to use vehicles other than retroviruses to genetically engineer or modify stem cells. Genetic information of interest can be introduced by means of any virus which can express the new genetic material in such cells. For example, SV40, herpes virus, adenovirus and human papilloma virus can be used for this purpose. Many other methods can also be used for introducing cloned eukaryotic DNAs into cultured mammalian cells, several of which are discussed below. The genetic material to be transferred to stem cells may be in the form of viral nucleic acids, bacterial plasmids or episomes. The latter have the advantage of extracellular nephication both in vitro and in vivo.

One of the most widely used methods is transfection mediated by either calcium phosphate or DEAE-dextran. It is believed that the transfected DNA enters the cytoplasm of the cell by endocytosis and is transferred to the nucleus. Up to 20% of a population of cultured cells can be transfected at any one time. Because of its high efficiency, transfection mediated by calcium phosphate or DEAE-dextran is another method of choice for experiments that require expression of the foreign DNA in large numbers of cells. Calcium phosphate-mediated transfection is also used to establish cell lines that carry

integrated copies of the foreign DNA, which are usually
arranged in head-to-tail tandem arrays.

The polycation Polybrene allows the efficient and stable
introduction of low molecular weight DNAs (e.g., plasmid DNAs)
into cell lines (e.g., CHO cells) that are relatively
resistant to transfection by other methods (Kawai and
Nishizawa 1984; Chaney et al. 1986).

Protoplasts derived from bacteria carrying high copy numbers
of copies of a plasmid of interest can be mixed directly with
cultured mammalian cells. After fusion of the cell membranes
(usually with polyethylene glycol), the contents of the
bacteria are delivered into the cytoplasm of the mammalian
cells and the plasmid DNA is transferred to the nucleus.
Protoplast fusion is not as efficient as transfection for many
of the cell lines that are commonly used for transient
expression assays, but it is useful for cell lines in which
endocytosis of DNA occurs inefficiently. Protoplast fusion
frequently yields multiple copies of the plasmid DNA tandemly
integrated into the host chromosome (Robert de Saint Vincent
et al. 1981). (Schaffner 1980; Rassoulzadegan et al. 1982).

In electroporation the application of brief, high-voltage
electric pulses to a variety of cells leads to the formation
of nanometer-sized pores in the plasma membrane (Neumann et
al. 1982; Zimmermann 1982). DNA is taken directly into the
cell cytoplasm either through these pores or as a consequence
of the redistribution of membrane components that accompanies
closure of the pores. Electroporation can be extremely
efficient and can be used both for transient expression of
cloned genes and for establishment of cell lines that carry
integrated copies of the gene of interest. Electroporation, in
contrast to calcium phosphate-mediated transfection and
protoplast fusion, frequently gives rise to cell lines that
carry one, or at most a few, integrated copies of the foreign
DNA (Boggs et al. 1986).

Artificial membrane vesicles (liposomes) are being intensively
studied for their usefulness as delivery vehicles in vitro and
in vivo. For a review of the current procedures for liposome
preparation, targeting, and delivery of contents, see Mannino
and Gould-Fogerite (1988). Most of these procedures involve
encapsulation of DNA or RNA within liposomes, followed by
fusion of the liposomes with the cell membrane. However,
Feigner et al. (1987) have reported that DNA that is coated
with a synthetic cationic lipid can be introduced into cells
by fusion. Although this method is simple and appears to be
efficient, it is comparatively new and untested (but see
Felgner and Holm 1989; Maurer 1989).

Although direct microinjection into nuclei has the advantage
of not exposing DNA to cellular compartments such as low pH
endosomes, it cannot be used to introduce DNA on a scale large
enough for biochemical analysis. Microinjection is therefore
used primarily as a method to establish lines of cells that
carry integrated copies of the DNA of interest. (Capecchi

1980).

The present invention makes it possible to genetically
engineer mesenchymal human stem cells in such a manner that
they produce polypeptides, hormones and proteins not normally
produced in human stem cells in biologically significant
amounts or produced in small amounts but in situations in
which overproduction would lead to a therapeutic benefit.
These products would then be secreted into the bloodstream or
other areas of the body, such as the central nervous system.
The human stem cells formed in this way can serve as a
continuous drug delivery systems to replace present regimens,
which require periodic administration (by ingestion,
injection, depot infusion etc.) of the needed substance.

For example, it can be used to provide continuous delivery of
insulin, which at present must be isolated from the pancreas
and extensively purified or manufactured in vitro
recombinantly and then injected into the body by those whose
insulin production or utilization is impaired. In this way,
insulin can be introduced into the body via a continuous drug
delivery system and, as a result, there would be no need for
daily injections of insulin.

Genetically engineered human mesenchymal stem cells can also
be used for the production of clotting factors. Hemophiliacs
lack a protein called Factor VIII, which is involved in
clotting. Factor VIII is now administered by injection. Human
stem cells having genes encoding Factor VIII, can be used to
make a skin graft (human MSCs are present in the dermis) in
which they produce Factor VIII; as a skin graft, the tissue
secretes the factor into the bloodstream. Such cells can also
be used for continuous delivery of dystrophin to muscle cells
fro muscular dystrophy.

Incorporation of genetic material of interest into human stem
cells and other types of cells is particularly valuable in the
treatment of inherited and acquired disease. In the case of
inherited diseases, this approach is used to provide
genetically modified human stem cells and other cells which
can be used as a metabolic sink. That is, such human stem
cells would serve to degrade a potentially toxic substance.
For example, this could be used in treating disorders of amino
acid catabolism including the hyperphenylalaninemias, due to a
defect in phenylalanine hydroxylase; the homocysteinemias, due
to a defect in cystathionine .beta.-synthase. Other disorders
that could be treated in this way include disorders of amino
acid metabolism, such as cystinosis; disorders of membrane
transport, such as histidinurea or familial
hypecholesterolemia; and disorders of nucleic acid metabolism,
such as hereditary orotic aciduria. Human mesenchymal stem
cells of the present invention can also be used in the
treatment of genetic diseases in which a product (e.g., an
enzyme or hormone) normally produced by the body is not
produced or is made in insufficient quantities. Here, human
stem cells transduced with a gene encoding the missing or
inadequately produced substance can be used to produce it in

sufficient quantities. This can be used in producing alpha-1 antitrypsin. It can also be used in the production of Factor XIII and Factor IX and thus would be useful in treating hemophilia. For any of these examples, includes in the present invention is the use of tissue specific promoters that allow increased expression in particular mesenchymal cell lineages and cells which would be used to limit gene expression into either the differentiated or precursor stem cell. Examples of such tissue-specific promoters include but are not limited to the promoter for the collagen type I genes or another collagen gene family, the promoter for the dystrophin gene and the promoter for stem cell factor.

There are many acquired diseases for which treatment can be provided through the use of engineered human stem cells (i.e., human stem cells transduced with genetic material of interest). For example, such cells can be used in treating anemia, which is commonly present in chronic disease and often associated with chronic renal failure (e.g., in hemodialysis patients). In this case, human stem cells having incorporated in them a gene encoding erythropoietin would correct the anemia by stimulating the bone marrow to increase erythropoiesis (i.e. production of red blood cells). Other encoded cytokines can be G-CSF or GM-CSF, for example.

Human stem cells of the present invention can also be used to administer a low dose of tissue plasminogen activator as an activator to prevent the formation of thrombi. In this case, human stem cells having incorporated genetic material which encodes TPA would be transplanted into an individual in whom thrombus prevention is desired. This would be useful, for example, as a prophylactic against common disorders such as coronary artery disease, cerebrovascular disease, peripheral vascular occlusive disease, vein (e.g., superficial) thrombosis, such as seen in pulmonary emboli, or deep vein thrombosis. Human stem cells which contain DNA encoding calcitonin can be used in the treatment of Paget's Disease, a progressive, chronic disorder of bone metabolism, in which calcitonin is presently administered subcutaneously.

Another application is a subcutaneous implatation of stem cells alone or adhered to a porous ceramic cube device which will house the stem cells and allow them to differentiate in vivo. Another example would be injection of stem cells into muscle where they will differentiate into muscle cells. An example might be a graft having genetically engineered human stem cells is in birth control. Tests are underway now for using a polypeptide hormone called lutenizing hormone releasing hormone (LHRH) in regulating fertility. Continuous administration of LHRH results in a sterile individual; when administration ceases, the individual is again fertile. Rather than taking LHRH injections or oral medication, one could have a small graft which continuously secretes LHRM to provide the same effect. In the event that the person wanted to regain fertility this transplant could be excised; delivery of the polypeptide hormone would cease.

Human stem cells engineered to produce and secrete interleukins (e.g., IL-1, IL-2, IL-3 or IL-4 through IL-11) can be used in several contexts. For example, the result of some of the therapies now used (e.g., chemotherapy) is induction of neutropenia (the presence of abnormally low numbers of neutrophils in the blood), often caused by direct suppression of the bone marrow. For example, use of virtually all the chemotherapeutic agents, results in neutropenia. This condition results in numerous life-threatening infections. In these cases, administration of, for example, IL-3 through transplantation of human stem cells which contain genetic material encoding IL-3 can be used to increase the neutrophil count. In addition, the administration of thrombopoietin, which stimulates the production of platelets, can be used in the treatment of numerous conditions in which platelet count is low. In this case, human stem cells transduced with the gene for thrombopoietin can be applied to an individual; production and secretion of the encoded product will result in stimulation of platelet production.

Another use of the present invention is in the treatment of enzyme defect diseases. In this case the product (polypeptide) encoded by the gene introduced into human stem cells is not secreted (as are hormones); rather, it is an enzyme which remains inside the cell. There are numerous cases of genetic diseases in which an individual lacks a particular enzyme and is not able to metabolize various amino acids or other metabolites. The correct genes for these enzymes could be introduced into the stem cells and transplanted into the individual; the transplant would then carry out that metabolic function. For example, there is a genetic disease in which those affected lack the enzyme adenosine deaminase. This enzyme is involved in the degradation of purines to uric acid. It is believed possible, using the present invention, to produce a subcutaneous graft as described above capable of producing the missing enzyme at sufficiently high levels to detoxify the blood as it passes through the area to which the graft is applied.

Additional uses not previously possible or suggested include but are not limited to:

1. cytokine genes to enhance hematopoietic reconstitution following marrow transplantation for bone marrow failure for congenital disorders of the marrow

2. bone cytokines to improve repair and healing of injured bone

3. bone matrix problems to improve repair and healing of injured or degenerative bone

4. correction of mesenchymal genetic disorders such as osteogenic imperfecta and muscular dystrophy

5. localized production of proteins, hormones etc. providing cellular therapeutics for many different compounds

6. cytotoxic genes such as thymidine kinase which sensitizes cells to ganciclovir. Gap junction adhesion to tumor cells could allow MSCs to serve for cancer therapy.

The present invention also has veterinary applications. It can be used, for example, in delivering substances such as drugs (e.g., antibiotics) and hormones to animals, which would otherwise be provided by being incorporated into their feed, added to their water or injected periodically (e.g., daily or less frequently). Use of the modified human stem cells of the present invention has the advantage that the tissue formed of the modified cells can be applied to the animal and will provide quantities of the encoded protein on an ongoing basis, thus eliminating the need for daily/periodic administration of the substance.

This invention also has industrial applicability in providing hormones, enzymes and drugs to mammals, including humans, in need of such substances. For example, it can be used to provide a continuous supply of a protein or polypeptide which otherwise would be intermittently administered intravenously, intramuscularly or subcutaneously. It is particularly valuable in providing such substances, such as hormones (e.g., parathyroid hormone, insulin), which are needed in sustained doses for extended periods of time.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed and exemplified by the following.

DETAILED DESCRIPTION:

EXAMPLE 1

Construction of vM5neolacZ

The vM5neolacZ retroviral vector was constructed in the following manner as described by Clapp et al. (4). Plasmid pM5neo includes the retroviral sequences of the murine Myeloproliferative Sarcoma Virus (MPSV), modified and constructed as described (9) to contain genetic sequences of the 5' and 3' long terminal repeats [LTR] from the MPSV virus (16, 19, 22), denoted in FIG. 1 as "MPSV", splice donor [sd] and splice acceptor [sa] sites from the parent MPSV virus, and the Tn5 neogene from the bacterial transposon 5 which encodes the neomycin resistance gene. When expressed, the neogene confers resistance to the neomycin analogue, G418 (20). These sequences were ligated into the BglII-HindIII site of pBR322 as described (9). The viral region of the plasmid contains a polylinker region of DNA encoding recognition sites for a number of restriction endonucleases of which Bam Hl and Eco Rl are uniquely represented within the viral and plasmid sequence. The U3 region of the MPSV viral 3' LTR contains

mutations which allow increased expression within precursor cells such as embryonic stem cells (18, 19, 22) and hematopoietic progenitors (4, 6). The intact sequences for the packaging recognition signal [Psi] required for packaging of these sequences into a retrovirus are 3' to the 5' LTR [see below]. The retroviral sequences contained within the pM5neo plasmid lack the genes essential for retroviral replication and thus is replication defective, as described (McLaughlin et al., supra).

The sequences for the lacZ gene, which encodes the bacterial gene for .beta.-galactosidase, were removed from the pMuMLV-SV-lacZ plasmid (10, 18) after sequential restriction enzyme digestion with Hind III and Bam Hl using standard molecular biology techniques (4,11). This yielded a 3.7 Kb fragment containing the lacZ gene. The 5' and 3' overhanging ends of the lacZ gene fragment were then made blunt by reaction with the Klenow fragment of bacterial DNA polymerase I and then both ends of the fragment were ligated to short sequences of DNA containing recognition sites for the Bam Hl restriction endonuclease [termed Bam Hl linkers] using T4 DNA ligase and standard molecular biology techniques (4,11).

To insert the lacZ gene into the pM5neo retroviral backbone, the plasmid was digested with BamHl restriction endonuclease and treated with phosphatase and the 3.7 Kb DNA fragment consisting of the lacZ gene with Bam Hl linkers at both the 3' and 5' ends was then ligated to pM5neo using DNA ligase by standard molecular biology techniques (4, 11). Following ligation, the plasmid was transformed into DH5alpha cells. Individual transformants were grown, harvested, and analyzed using the method of colony hybridization described by Grunstein (8). Orientation of transformants were confirmed by digestion with Eco Rl restriction endonuclease. The expected 12.3 and 0.4 Kb fragments [see FIG. 1] were observed in multiple colonies. This placed the lacZ gene 5' to the neo gene within the retroviral sequences. mRNA transcripts derived from the LTR of this retrovirus will generate two products: the intact mRNA containing lacZ and neo genes and a spliced mRNA which contains sequences only for the neogene.

Retroviral plasmid pM5neolacZ was isolated and purified from the bacteria by standard techniques (11), and transfected into the GP.sup.+ envAm-12 [Am-12] retrovirus packaging cell line (12, 13) by Lipofectin Reagent.TM. according to the instructions of the manufacturer [BRL-GIBCO]. The Am-12 cell line is a helper-free retrovirus producer cell line which was created by transfecting into NIH-3T3 cells, three plasmid DNA sequences. The first one of which contains the retroviral sequences for gag and pol [plasmid pgagpolgpt]. Gag encodes the nucleocapsid protein and pol encodes the retroviral reverse transcriptase both of which are essential for the life cycle of infectious retrovirus as described by McLaughlin, et al. supra). Gpt encodes for a protein which induces resistance to mycophenolic acid and serves as a selectable gene for enrichment of cells carrying the genetic sequences (12, 14). Following electroporation of this plasmid into NIH-3T3 cells,

cells were selected for those expressing high levels of the
gpt gene in medium containing hypoxanthine (15 .mu.g/ml),
xanthine (250 .mu.g/ml) and mycophenolic acid (25 .mu.g/ml)
and for high expression of reverse transcriptase (12, 13).

The selected cell clones were co-electroporated with the
second and third plasmids: penv-Am, which contains the nucleic
acid sequences for the amphotropic envelope protein, env-Am,
which, when expressed on the surface of a retrovirus, has a
host range of both murine, primate and human cells; and
pRSVhyg which contains the nucleic acid sequences for the hyg
gene which, when expressed in recipient cells, induces
hygromycin resistance (7). Plasmids penv-Am12 and pgagpolgpt
contain nucleic acid sequences removed from the 3PO plasmid
(14, 15) which includes sequences from the MoMuLV retrovirus
beginning at the 5' end of the known sequence including the 5'
LTR region encoding the promoter-enhancer region of the MoMuLV
which drives expression of the linked genetic sequences [gag,
pol, gpt in pgagpolgpt or env in penv-Am] and a deletion of
the packaging site region of the MoMuLV.

The advantage of this set of plasmids is that the genes
essential for wild-type [normal] function of the MoMuLV are
located on two different plasmids and transfected separately
into the NIH-3T3 cells. Furthermore, as described above, only
after transfection of the replication defective virus are the
packaging [Psi] sequences introduced into the cell. Thus, it
is unlikely that three different recombinational events will
take place between these regions of retroviral sequences in
the integrated DNA of the cell to yield sequences which encode
an intact infectious retrovirus, and this event has yet to be
reported with the Am-12 cell line. As a result, this cell line
produces high levels of protein products from the gag, pol and
env-Am genes but no infectious retrovirus (12, 13).

Following transfection with pM5neolacZ, the Am-12 cells were
grown to confluence in a culture medium which consisted of 50%
Dulbecco's Modified Eagle Medium [DMEM] plus 50% F-12 medium
supplemented with 7% FCS and 3% calf serum, 1%
penicillin-streptomycin solution, 2 mM glutamine and 15 mM
HEPES buffer [complete medium]. Heat inactivated (HI) serum
[prepared by incubation at 56.degree. C. for 40 minutes] was
used to avoid inadvertent inactivation of retrovirus by
complement. Thereafter, cultured cells were trypsinized and
diluted to allow growth of individual clones while being grown
in complete medium in the presence of the neomycin analogue
G418 at a concentration of 1 mg/ml. After selection, surviving
cells were grown either in the absence of G418 for collection
of supernatant containing virus or in 0.2 mg/ml G418 for
maintenance of the transduced genes. Supernatant culture
medium from confluent cultures was collected, placed at
4.degree. C., passed through a 0.22 micron sterile filter to
remove remaining cells but to allow recovery of vM5neolacZ
retroviral particles, and stored at -80.degree. C. or used
immediately.

Supernatant from these cells was used to infect the ecotropic

producer cell line, GP.sup.+ E.sup.- 86. The GP.sup.+ E.sup.-
86 cell line is a helper-free retrovirus producer cell line
similar to the Am-12 cell line described above. The GP.sup.+
E.sup.- 86 cell line was created by cotransfecting, using
electroporation, into NIH-3T3 cells, the plasmid pgagpolgpt
[see above] and the plasmid penv which contains the nucleic
acid sequences, env, encoding the ecotropic envelope protein
(13). Supernatant from the vM5neolacZ-infected GP.sup.+
E.sup.- 86 cell line culture was then used to reinfect the
AM12 cells originally transfected with pM5neolacZ. In-each
instance, the infection protocol was identical and cells were
selected in G418 at 1 mg/ml.

Clone Am12-lacZ2 (also referred to as "PNL-2") was selected
because it gave a high titer of retrovirus quantitated by the
ability to transfer G418 resistance and lacZ expression to
NIH-3T3 cells using methods previously described (4b).
Briefly, supernatant from individual clones of the transduced
Am-12 packaging cell line was collected as described above and
the concentration of retrovirus quantitated by infecting
$2.times.10.sup.5$ NIH-3T3 cells which had been adhered to 100
mm.sup.2 dishes 24 hrs previously. The infections were done in
a total volume of 1 ml of medium containing limiting dilutions
of supernatant [0.01 to 1 .mu.l/plate], 8 .mu.g/ml of
Polybrene and 10% heat inactivated fetal calf serum (HI FCS)
and dishes were incubated at 37.degree. C. After a six-hour
incubation, 10 ml complete medium was added and the growth of
the NIH-3T3 cells was allowed to continue until the plates
were confluent. Individual clones were analyzed for
.beta.-galactosidase activity by staining the retrovirally
infected NIH-3T3 cells with 1 mg/ml of X-gal substrate by
standard techniques (10). No detectable recombinant wild-type
retrovirus was present in the viral supernatant used in these
experiments as analyzed by the following experiments. First,
NIH-3T3 cells were infected with retroviral supernatant and
allowed to grow to confluence. Media from the NIH-3T3 cells
was unable to produce G418 resistant colonies when used to
infect a second plate of NIH-3T3 cells. Secondly, no provirus
was detected in the secondary infections as analyzed by
amplification of the neo or lacZ gene. Third, this secondary
supernatant could not induce ["rescue"] the release into the
supernatant of provirus integrated into NIH-3T3 cells
previously infected with the vM5neolacZ virus. Fourth, serum
of recipient animals did not contain infectious helper
retrovirus when assayed.

Am12-lacZ cells were grown in culture for periods of up to 10
weeks after which early passage cells that had previously been
cryopreserved were thawed and used for subsequent passage.
Cells were grown in DMEM 50%/F12 50% complete medium, with 7%
fetal calf serum and 3% calf serum. To begin virus collection,
confluent cells, placed in 100 mm dishes, had removal of
culture medium and its replacement by 7 ml of fresh DMEM/F12
with 10% HI FCS. Cells were cultured at 37.degree. C. for 24
hours and medium collected daily for six days. The highest
titer of virus was usually collected between days 3 and 6.
Retroviral titers of $5-10.times.10.sup.5$ CFU/ml [colony

forming unit] as defined above. The titer was confirmed for
each batch of retroviral supernatant and batches were selected
in which the target NIH-3T3 cells which grew in the presence
of G418 also expressed the lacZ gene and turned blue following
incubation of fixed cells with X-gal. In most instances, cells
transduced with the supernatant from Am12-PNL2 cells and
selected for G418 resistance were expressing lacZ.

EXAMPLE 2

Description of the Genetic Transduction of MSCs

Human MSCs were cultured from adult bone marrow aspirates as
previously described (U.S. Pat. Nos. 5,197,985 and 5,226,914).
Normal donors or patients with normal bone marrow undergoing
autologous bone marrow harvest in conjunction with treatment
for a malignancy (9a). Ten ml bone marrow aspirates were
separated by Percoll gradient centrifugation and the
mononuclear cells were cultured on plastic tissue culture
dishes in DMEM medium with 10% fetal calf serum as previously
described (U.S. Pat. No. 5,226,914, (8a)). Similar results
were obtained substituting 50% DMEM/50% F-12 medium for DMEM
medium. These conditions allow selective attachment of
marrow-derived mesenchymal cells which retain the
multipotential capacity to differentiate along a number of
pathways as previously described (8a,8b). Three days later,
nonadherent cells were removed and fresh complete medium was
added to the cells.

At first passage, 1-3.times.10.sup.5 cells were placed on
plastic tissue culture dishes at a density of approximately
30% confluence in complete medium [DMEM 50%/F12 50% with 30%
HI FCS. As noted in Example 1, the HI FCS lacks complement
which could inactivate retrovirus upon contact. Four hours
after passage, the cells were retrovirally infected as
follows: a) medium was removed from the culture dishes; b) 5
ml of viral supernatant from Am-12PNL2 cells [which contains
the retrovirus vM5neolacZ as described above at a titer of
5-10.times.10.sup.5 cfu/ml]. The supernatant was prepared as
described above, and was added in the presence of 6 .mu.g/ml
Polybrene; c) after a 6 hr incubation at 37.degree. C., 5 ml
complete medium with 30% HI FCS was added. This procedure was
repeated twice on a daily basis. The medium of the culture
dishes was changes every 3-4 days and the cells were allowed
to grow to 80% confluence, trypsinized to remove them from the
plastic dishes as previously described and were split 1:3 into
new dishes.

After the third infection with retroviral supernatant, some
cultures were exposed to 0.5 mg/ml G418 to select for cells
expressing the neogene, using a technique previously described
(20), and others were grown to confluence on the dishes.
Assessment of cells transduced by this process was made by the
level of G418 selection and lacZ expression prior to selection
in G418. FIG. 2 shows that 6-30% of the cultured MSCs had
evidence of retroviral transduction prior to selection as
measured by staining blue after exposure to X-gal) and that

three infections was more efficient than one. Cells not
carrying the transduced neogene were killed by as little as
0.075 mg/ml G418. Selected cells were allowed to grow to 80%
confluence in the presence of 0.5 mg/ml G418 (FIG. 3).
Thereafter, they were grown in 0.2 mg/ml G418 with medium
change every 3-4 days and were passaged when the cultures
became 40-90% confluent by dividing the trypsinized cells 1:3
into new culture dishes. Three to four passages were routinely
performed with maintenance of proliferation of the cells and
without evidence of differentiation or loss of the mesenchymal
phenotype as outlined as 1) to 3) above. At various time
points, cells were evaluated for expression of the lacZ gene
(FIG. 4) as described below.

Their status as MSCs was confirmed by the following: 1)
morphology: cells were fusiform adherent cells with multiple
projections without round cell contamination (8a, 8b); 2)
surface antigens: cells bound to the SH2, SH3 and SH4
monoclonal antibody which recognize human mesenchymal stem
cells using techniques previously described (8b); 3) colony
formation in methylcellulose: cells were also noted to have an
absence of hematopoietic progenitor cell contamination as
indicated by the lack of growth of hematopoietic colonies
[colony forming unit-granulocytel/macrophage, CFU-GM; colony
forming unit erythroid, CFU-E; burst forming unit-erythrox,
PFU-E; or colony forming unit granulocyla, macrophage,
erythroid, megakaryocyte, CFU-GEMM] when 1.times.10.sup.5 of
these cells were grown in methylcellulose in the presence of
recombinant human hematopoietic growth factors including
erythropoietin, IL-3, GM-CSF and Stem Cell Factor, using
techniques previously described (9a).

Similar experiments were performed with marrow-derived MSCs
from rat and mouse. Mononuclear cells flushed from the marrow
cavities of Fisher rats and CeH/HeJ mice were cultured for
MSCs as previously described (4c) and passaged when 60-80%
confluent. Cells were infected with retrovirus as described
above for human MSCs except that ecotropic virus derived from
the GP.sup.+ E.sup.- 86 cell line was used. A similar efficacy
of gene transduction and expression of both the neo and lacZ
genes was noted in these cells as for human MSCs, ranging from
2-10% of unselected cells. Following selection in 0.5 mg/ml
G418, MSC cultures containing uniformly G418 resistant were
observed. In these cultures, 50-80% of the cells also
expressed the lacZ gene and stained blue after exposure to
X-gal reagent (see below). Thus, MSCs from three species have
been retrovirally transduced, selected in G418, and culture
expanded.

To test the ability of these cells to retain their mesenchymal
stem cell phenotype, cells were collected by trypsinization as
previously described (8c), centrifuged and resuspended in
serum free medium and co-incubated with biphasic ceramic cubes
composed of a mixture of tricalcium phosphate and
hydroxyapatite (60:40) with a mean pore size of 200 .mu.m. The
ceramics were cut into 3.times.3.times.3 mm cubes and coated
with human fibronectin prior to incubation. After incubation,

approximately 5.times.10.sup.4 cells adhere to each ceramic cube, as previously described (8a,4c). These coated cubes were then implanted under sterile conditions subcutaneously into the lateral flank of SCID mice, where they were allowed to stay for up to 8 weeks as previously described (4c).

At various time points after culture in vitro and 3, 6 or 8 weeks after being placed in vivo, the cells on plates or cells in cubes were washed twice in PBS and fixed in 1% glutaraldehyde and stained for .beta.-galactosidase with X-gal as previously described (10). Briefly, the cells and cubes were maintained at 22.degree. C. rinsed in 1.times. PBS and fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS at 4.degree. C. (cells for 5 mins., cubes for 45 mins.). Both were then rinsed three times in 1.times. PBS (cubes were incubated in PBS 3 times for 20 mins.). They were stained overnight in 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS at room temperature (Sanes et. al., EMBO 5:3133, 1986). Blue stain within the cytoplasm of cells were detected on the plates by direct visualization and in the cubes after decalcification and section as previously described (4c). Cells on plates and the cell nuclei in the sections were counter-stained with neutral red or crystal violet stain. Cubes were also stained with Mallory-Heidenhain stain to detect the presence of osseous bony material.

The results obtained indicated that between 6-30% of primary human, rat or mouse MSCs in culture could be retrovirally transduced. The gene transfer efficiency was higher in human than mouse or rat due to either optimal growth conditions for the human MSCs or the properties of the amphotropic virus. This efficiency was defined as the percentage of cells staining with X-gal prior to selection in G418, or the proportion of colony forming cells which grew in the presence of 0.5 mg/ml G418 (FIGS. 2 and 3). A high proportion [40-90%] of cells expressing neo and resistant to G-418 also expressed lacZ and stained with X-gal (FIGS. 3 and 4). Presence of the provirus was confirmed by PCR amplification of the neo gene by standard techniques. Retroviral transduction by infection also had no demonstrable effect on cell growth in the absence of selection. Thus, human transduced MSCs could be culture-expanded through 4-7 passages (approximately 20-30 cell divisions) and still maintain their stem cell phenotype. Rodent MSCs, however, grew more slowly and could typically be passaged only 1-2 times before becoming quiescent, and did not expand if passaged more frequently.

When analyzed for the ability to retain their precursor phenotype after selection in G418 and culture expansion, the human MSCs were able to form bone in the ceramic cubes at 6 and 8 wks (FIG. 5). At 3-8 weeks, blue lacZ.sup.+ cells were detected lining the ceramic cubes and at 6-8 weeks, blue cells embedded within bony lacunae were seen. These results are shown in FIG. 5. The cells lining the calcium phosphate pores of the cube appear to be precursors to osteoblasts whereas those within the lacunae are osteocytes. The pores of the

ceramic became filled with host [mouse] connective tissue cells and vasculature, as observed previously (4a, 4c). As a control, NIH-3T3 cells were transduced with retrovirus and selected in G418. These cells were then adhered to the ceramic cubes and placed subcutaneously in the SCID mouse. At 6 weeks, clusters of fibroblastic cells were found within the ceramic pores without evidence of bone formation within the ceramic cubes.

These results indicated: a) that the precursor, genetically transduced MSCs expressed both the neo and lacZ genes when grown under selection pressure of G-418 in vitro; b) that these retrovirally transduced cells retained their "stem cell" phenotype, after in vitro passage for many weeks without evidence of differentiation into osteogenic cells or stromal bone marrow cells, as measured by their ability to differentiate into osteoblasts and osteocytes in vivo, and c) that even when no longer under the selection pressure of G418, these cells retained the ability to express a foreign gene in vivo for at least 8 weeks as they proliferate and pass through the differentiation process, i.e. the genetic transduction has become a stable part of the cellular, genomic DNA. As such, they are unique in being human mesenchymal stem cells derived from a non-fetal, or in this case, adult host which have the capacity to be transduced and culture expanded and have been shown to retain their precursor stem cell phenotype. While loss of the transduced genes occurred in some cells, the majority of cells appear to have retained the proviral genes after a period of prolonged growth in vivo.

EXAMPLE 3

Use of MSCs as Retrovital Producer Cells

Using the plasmid sequences outlined above in example 1, with the present technology, human MSCs can be transduced either by electroporation, lipofection or retroviral gene transfer, with the sequences required to transform these cells into a retroviral producer cell. Thus, human MSCs can be transduced with the plasmids pgagpolgpt, penvAm-12 and pRSVhyg to produce a cell or cell culture which functions much like Am-12. The advantages that the resulting cells have over the current Am-12 cells are the following: a) the cells are not immortalized; b) they can be used as a source of autologous cells for both in vitro and in vivo gene transfer once the replication defective retrovirus carrying the gene of interest is introduced into the cells; c) they can be used as an in vivo source of retrovirus production and thus of gene transfer; and d) they can be used as autologous virus producing cells for studies involving the in vitro or in vivo retroviral transduction of hematopoietic stem cells (which for instance are contained in autologous bone marrow), peripheral hematopoietic progenitors isolated from the blood typically after treatment with a hematopoietic growth factor or chemotherapy or the combination, or are isolated by a variety of procedures from these two sources by the use of physical or monoclonal antibody techniques as described by others. In

addition, other selectable genes could be used in the place of the gpt or the hyg gene which potentially offer advantages in the level of cell selection and the ease of use. These include the neogene described above, and other selection genes.

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CLAIMS:

What is claimed is:

1. Isolated human mesenchymal stem cells which can differentiate into more than one connective tissue type transfected with exogenous genetic material encoding a protein to be expressed.

2. The isolated human mesenchymal stem cells of claim 1 that are non-embryonic mesenchymal stem cells.

3. The isolated human mesenchymal stem cells of claim 1 that are marrow-derived mesenchymal stem cells.

4. The isolated human mesenchymal stem cells of claim 1 that have been transfected with a DNA sequence which codes for at

least one protein to be expressed.

5. The isolated human mesenchymal stem cells of claim 4 that have been transfected with a DNA sequence comprising a retroviral 5' LTR and at least one of a retroviral gag, pol or env gene under the transcriptional control of said 5' LTR.

6. The isolated human mesenchymal stem cells of claim 5 that have also been transfected with a DNA sequence comprising a retroviral packaging signal sequence.

7. The isolated human mesenchymal stem cells of claim 1 which include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH2, deposited with the ATCC under accession number HB10743.

8. The isolated human mesenchymal stem cells of claim 7 which further include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH3, deposited with the ATCC under accession number HB10744.

9. The isolated human mesenchymal stem cells of claim 7 which include a cell surface epitope specifically bound by antibodies from hybridoma cell line SH4, deposited with the ATCC under accession number HB10745.

10. A composition comprising the isolated human mesenchymal stem cells of claim 1 in a medium that stimulates their culture expansion but does not stimulate their differentiation.

11. The composition of claim 10 wherein the medium comprises a supplemented DMEM.

12. A composition of claim 11 wherein the medium comprises serum.

13. The composition of claim 12 wherein the medium comprises fetal animal serum.

14. The composition of claim 11 which is supplemented with an antibiotic and antimycotic composition.

15. A composition comprising the isolated human mesenchymal stem cells of claim 1 in supplemented BGJ.sub.b medium.

16. A composition comprising the isolated human mesenchymal stem cells of claim 1 in supplemented F-12 Nutrient Mixture.

17. The composition of claim 1 wherein the isolated human mesenchymal stem cells of claim 1 are isolated from other cells by selective binding with antibodies from hybridoma cell line SH2, deposited with the ATCC under accession number HB10743.

18. The composition of claim 10 wherein the mesenchymal stem cells are isolated from other cells by density gradient

fractionation.

19. The composition of claim 10 wherein the mesenchymal stem cells are isolated from other cells by Percoll gradient fractionation.

20. The isolated human mesenchymal stem cells of claim 4 that have been transduced with a retroviral vector that includes the DNA sequence that codes for the protein to be expressed.

21. The isolated human mesenchymal stem cells of claim 20 wherein transcription of the DNA sequence is under the control of a retroviral LTR .